Genetic diversity and biocontrol potential of fluorescent pseudomonads producing phloroglucinols and hydrogen cyanide from Swiss soils naturally suppressive or conducive to *Thielaviopsis basicola*-mediated black root rot of tobacco

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Abstract

Pseudomonas populations producing the biocontrol compounds 2,4-diacetylphloroglucinol (Phl) and hydrogen cyanide (HCN) were found in the rhizosphere of tobacco both in Swiss soils suppressive to *Thielaviopsis basicola* and in their conducive counterparts. In this study, a collection of Phl^+ HCN^+ *Pseudomonas* isolates from two suppressive and two conducive soils were used to assess whether suppressiveness could be linked to soil-specific properties of individual pseudomonads. The isolates were compared based on restriction analysis of the biocontrol genes *phlD* and *hcnBC*, enterobacterial repetitive intergenic consensus (ERIC)-PCR profiling and their biocontrol ability. Restriction analyses of *phlD* and *hcnBC* yielded very concordant relationships between the strains, and suggested significant population differentiation occurring at the soil level, regardless of soil suppressiveness status. This was corroborated by high strain diversity (ERIC-PCR) within each of the four soils and among isolates harboring the same *phlD* or *hcnBC* alleles. No correlation was found between the origin of the isolates and their biocontrol activity *in vitro* and *in planta*. Significant differences in *T. basicola* inhibition were however evidenced between the isolates when they were grouped according to their biocontrol alleles. Moreover, two main *Pseudomonas* lineages differing by the capacity to produce pyoluteorin were evidenced in the collection. Thus, Phl^+ HCN^+ pseudomonads from suppressive soils were not markedly different from those from nearby conducive soils. Therefore, as far as biocontrol pseudomonads are concerned, this work yields the hypothesis that the suppressiveness of Swiss soils may rely on the differential effects of environmental factors on the expression of key biocontrol genes in pseudomonads rather than differences in population structure of biocontrol *Pseudomonas* subcommunities or the biocontrol potential of individual Phl^+ HCN^+ pseudomonad strains.

Introduction

Many saprophytic microorganisms colonizing plant roots have the ability to protect plants from damage caused by parasitic nematodes and bacterial and/or fungal pathogens, and several strains have been used as biocontrol agents (Paulitz & Bélanger, 2001; Weller *et al*., 2002; Harman *et al*., 2004; Moënne-Loccoz & Défago, 2004; Haas & Défago, 2005). The main plant-beneficial modes of action include competition, antagonism and induced resistance (Bakker *et al*., 2003; Haas & Keel, 2003; Kloepper *et al*., 2004). In certain soils, indigenous plant-beneficial microorganisms are able to provide effective protection from certain phytopathogens/parasites, and these soils are referred to as 'disease suppressive' (Lemanceau & Alabouvette, 1993; Mazzola, 2002; Weller *et al*., 2002; Rimé *et al*., 2003).

In Morens, Switzerland, long-standing soil suppressiveness to black root rot of tobacco caused by *Thielaviopsis basicola* has been attributed to the antagonistic activity of fluorescent pseudomonads (Stutz *et al*., 1986). In one such strain, designated CHA0, hydrogen cyanide (HCN) (Voisard *et al*., 1989) and 2,4-diacetylphloroglucinol (Phl; Keel *et al*., 1992) have been shown to be major determinants of the

strain's biocontrol ability. HCN and, especially, Phl are also implicated in the biological control of soil-borne phytopathogens by other antagonistic fluorescent pseudomonads (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Keel *et al.*, 1992; Cronin *et al.*, 1997; Ellis *et al.*, 2000; Haas & Keel, 2003; Moënne-Loccoz & Défago, 2004). In addition, Phl^+ pseudomonads and Phl synthesis contribute to the monoculture decline of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Raaijmakers & Weller, 1998; Weller *et al.*, 2002; de Souza *et al.*, 2003).

Previously, we determined the abundance and diversity of *phlD*-harboring fluorescent *Pseudomonas* isolates from Morens suppressive and conducive soils (Ramette *et al.*, 2003a). In contrast to the situation found with take-all decline (Raaijmakers & Weller, 1998; Weller *et al.*, 2002), Phl^+ fluorescent pseudomonads were readily found in conducive soils, at population levels that were very similar to those in suppressive soils. Consequently, disease suppressiveness in Morens did not result from the higher population levels of these bacteria, as it did in the case of take-all decline. Therefore, if the hypothesis that suppressiveness is mainly due to antagonistic pseudomonads is still valid, it means that suppressiveness of Morens soils may result from the occurrence of (i) particular strain genotypes of HCN^+ Phl^+ fluorescent pseudomonads, and/or (ii) particular soil conditions promoting expression of biocontrol genes. At Morens, extensive *phlD* polymorphism was found in isolates from both types of soils (Ramette *et al.*, 2003a). However, only one biocontrol gene was assessed (despite the fact that Phl^+ pseudomonads were also HCN^+). In addition, whether populations of HCN^+ Phl^+ fluorescent pseudomonads differed in terms of (i) strain genetic diversity and (ii) the biocontrol ability of individual strains was not known. These questions are addressed in the present study.

Here, a collection of HCN^+ Phl^+ *Pseudomonas* isolates from both types of soils and displaying the main *phlD* alleles found at Morens (Ramette *et al.*, 2003a) was used for genetic and phenotypic comparisons. The first objective was to determine whether additional genetic differences could be revealed at a finer level of resolution. To this end, the allelic diversity of another biocontrol locus, i.e. *hcnBC* (encoding an HCN synthase), was examined in parallel to that of *phlD*, and the extent of strain diversity was determined by enterobacterial repetitive intergenic consensus (ERIC)-PCR. The second objective was to assess whether the functional differences between isolates correlated with the suppressive status of the soil they originated from. The biocontrol properties of the isolates were determined *in vitro* (inhibition of the pathogen *T. basicola*) and *in planta* (suppression of black root rot of tobacco). Plant protection by the reference *Pseudomonas* strain CHA0 was higher in the presence of iron-rich clay minerals (e.g. vermiculite) compared with iron-poor clay minerals such as illite (Keel *et al.*, 1989; Voisard *et al.*, 1989). Vermiculitic and illitic clay minerals predominate in Morens suppressive and conducive soils, respectively (Stutz *et al.*, 1989). Therefore, biocontrol experiments *in planta* were carried out using artificial vermiculitic and illitic soils mimicking the physico-chemical properties of Morens suppressive and conducive soils, respectively.

Materials and methods

Pseudomonas collection

The 30 *Pseudomonas* isolates (Table 1) were obtained by Ramette *et al.* (2003a) from the rhizosphere or macerated roots of tobacco exposed to *Thielaviopsis basicola* and grown in greenhouse pots containing sandy loam soil (Ramette *et al.*, 2003a) from Morens (western Switzerland) conducive (soils MC6 and MC10) or suppressive (soils MS7 and MS8) to black root rot of tobacco (four tobacco plants per soil). They correspond to a representative subset of 52 isolates (Ramette *et al.*, 2003a) and were selected because they harbored prevalent *phlD* alleles (i.e. AAAA, FBDC, JDCC, DCCC, HBNC, HBDC; Table 2) that were associated with both suppressive and conducive soils (Ramette *et al.*, 2003a). The bacteria were routinely grown on King's B agar (King *et al.*, 1954) at 27 °C and stored at –80 °C in 40% glycerol. *T. basicola* (Berk. and Br.) Ferraris strain ETH D127 was grown on malt agar (Difco Laboratories, Detroit, MI) at 24 °C.

Production of key biocontrol metabolites

All *Pseudomonas* isolates are Phl^+ and HCN^+ (Ramette *et al.*, 2003a). Here, the ability of the isolates to produce *in vitro* the Phl precursor monophloroglucinol and pyoluteorin (Plt) was determined by high-performance liquid chromatography, as described elsewhere (Notz *et al.*, 2001). Secondary metabolite production was determined twice.

Restriction analysis of *phlD* and *hcnBC* PCR amplicons

PCR amplification, restriction analyses and pattern classification of *phlD* and *hcnBC* were done as reported previously (Ramette *et al.*, 2001, 2003a,b; Wang *et al.*, 2001). Briefly, the restriction analysis of amplicons was performed using either *Hae*III, *Mnl*I, *Msp*I, *Nde*II or *Sau3*A (Boeringer, Mannheim, Germany) at least twice for each isolate. The presence and absence of bands in restriction profiles were used to generate a similarity matrix using the Dice coefficient (Dice, 1945). The similarity matrix was clustered using the unweighted pair-group method with arithmetic mean (UPGMA), and nodal support was assessed by performing 1000 bootstrap resamplings using Winboot (Yap & Nelson, 1996).

Table 1. Origin of the Phl$^+$ fluorescent *Pseudomonas* strains used in the study

Pseudomonas isolates		Host plant*	Biocontrol activity†	Soil origin: Designation and/or property	Soil origin: Geographic location	Reference
Morens isolates‡						
C6-	2, 7, 8, 9, 11, 16, 17, 23	Tobacco	Tobacco (Tb)*	MC6, conducive to Tb (tobacco)	Morens, Switzerland	Ramette *et al*. (2003a)
C10-	181, 186, 189, 190, 197, 204, 205	Tobacco	Tobacco (Tb)*	MC10, conducive to Tb (tobacco)	Morens, Switzerland	Ramette *et al*. (2003a)
S7-	29, 42, 46, 48, 49, 51, 52	Tobacco	Tobacco (Tb)*	MS7, suppressive to Tb (tobacco)	Morens, Switzerland	Ramette *et al*. (2003a)
S8-	62, 78, 110, 130, 151, 153, 154, 159	Tobacco	Tobacco (Tb)*	MS8, suppressive to Tb (tobacco)	Morens, Switzerland	Ramette *et al*. (2003a)
Reference strains						
CHA0		Tobacco	Tobacco (Tb), wheat (Ggt), cucumber (Pu)	MS1, suppressive to Tb (tobacco)	Morens, Switzerland	Ramette *et al*. (2003a)
Pf-5		Cotton	Cotton (Pu, Rs), cucumber (Pu)	NA	Texas, USA	Howell & Stipanovic (1979)
Q2-87		Wheat	Wheat (Ggt)	Take-all suppressive soil (wheat)	Quincy, Washington, USA	Vincent *et al*. (1991)
Q65c-80		Wheat	Wheat (Ggt)	Take-all suppressive soil (wheat)	Quincy, Washington, USA	Harrison *et al*. (1993)
F113		Sugar beet	Sugar beet (Pu), potato (Eca)	NA	Ireland	Fenton *et al*. (1992)
PITR2		Wheat	Cucumber (Pu), tomato (FORL)	Soil suppressive to Fusarium wilt (tomato)	Albenga, Italy	Keel *et al*. (1996)

*All biocontrol pseudomonads were isolated from the rhizosphere, macerated roots or roots previously washed of soil.
†Bicontrol activity established in the present study (*); Tb, *Thielaviopsis basicola*; Ggt, *Gaeumannomyces graminis* var. *tritici*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; Ps, *Phomopsis sclerotioides*; St, *Septoria tritici*; Eca, *Erwinia carotovora* subsp. *carotovora*.
‡The name of Morens isolates (e.g. C6-2) includes a prefix indicating the soil of origin (e.g. C6 for soil MC6) and an identification number (e.g. 2 for isolate C6-2).
NA, not available.

ERIC-PCR analysis

Primers and conditions used to perform ERIC-PCR were described by Rademaker *et al*. (1998). PCR products were separated on 1.5% agarose gel and the band sizes were evaluated by comparison with a 1-kb DNA ladder (Gibco-BRL, Basel, Switzerland) using GelCompar II software (version 3.0, Applied Maths, Austin, TX). The pairwise similarity between ERIC-PCR patterns was determined using the Dice coefficient, and cluster analysis was done using UPGMA. For each isolate, the experiment was done at least twice and data indicated that the profiles were at least 95% similar. Therefore, 95% was chosen as the minimal cut-off value to define unique ERIC-PCR genotypes in Fig. 3.

Analysis of biocontrol potential *in vitro* and *in planta*

For each *Pseudomonas* isolate, three 10 μL spots (about 10^7 CFU per spot) and a 2-week old, malt agar plug of *T. basicola* were placed simultaneously on to malt agar. The distance between the bacterial spots and the fungal plug was 2 cm, and the distance between the fungal and bacterial colonies was measured after 10 days incubation at 24 °C. For each strain tested, this inhibition distance was expressed as a ratio, by dividing the data by that for the reference biocontrol strain *Pseudomonas* sp. CHA0 (isolated from Morens) within each replicate. The experiment was repeated twice, and data from the three experiments were combined, as interexperimental variation was not significant (data not shown).

Biocontrol activity against *T. basicola in planta* was assessed under gnotobiotic conditions, using standardized artificial soil systems that mimicked either a suppressive or a conducive soil from Morens by the addition of vermiculitic or illitic clay minerals, respectively (Keel *et al*., 1989; Stutz *et al*., 1989; Voisard *et al*., 1989). Each treatment was studied using a sterile transparent container (Phytatray, Sigma, St. Louis, MO) containing 200 cm^3 sterile artificial soil (Keel

Table 2. Genotypic characteristics of Morens fluorescent pseudomonads

	Restriction of biocontrol genes		ERIC-PCR[†]		Estimated abundance in Morens soil[‡]
Isolate	*phlD*	*hcnBC*	Genotype	Cluster	Log CFU (g root)$^{-1}$
From conducive soils					
C6-2◆.[§] C6-23 ◆	AAAA	AGMY	A	I	6.9 ± 0.2
C6-11 ◆	AAAA	AGMY	J	III	6.9 ± 0.2
C6-9, C6-16	FBDC	CJMc	D	III	6.9 ± 0.2
C10-181	JDCC	FLOa	L	III	6.0 ± 0.2
C10-186	DCCC	AKOb	U	VII	6.1 ± 0.2
C10-189	DCCC	AKOb	T	VI	6.1 ± 0.2
C10-190	DCCC	AKOb	Q	VI	6.1 ± 0.2
C10-205	DCCC	AKOb	R	VI	6.1 ± 0.2
C10-197	HBNC	ELOa	N	IV	5.5 ± 0.2
C10-204	HBDC	AfOa	M	IV	5.5 ± 0.2
From suppressive soils					
S7-29 ◆	AAAA	AGMY	H	III	5.7 ± 0.2
S7-52 ◆	AAAA	AGMY	I	III	5.7 ± 0.2
S7-42	FBDC	CJMc	K	III	6.3 ± 0.2
S7-46	FBDC	CLMc	E	III	6.3 ± 0.2
S8-151	FBDC	CJMc	G	III	6.3 ± 0.2
S8-62 ◆	AAAA	AGMY	A	I	6.7 ± 0.2
S8-130 ◆	AAAA	AGMY	O	IV	6.7 ± 0.2
S8-110	HBNC	ELOa	M	IV	6.3 ± 0.2
Reference strains					
CHA0 ◆	AAAA	AGMY	A	I	NA
Pf-5 ◆	AAAA	AGMY	B	I	NA
PITR2	DCCC	DKOb	S	VI	NA
Q2-87	BBBB	BHNZ	P	V	NA
Q65c-80	FBDC	CJMc	F	III	NA
F113	GBCC	BIMa	C	II	NA

The four-letter code corresponds to the restriction patterns successively obtained with *Hae*III, *Cfo*I, *Msp*I, and *Nde*II, as defined previously (Ramette *et al*., 2003a; Wang *et al*. 2001). For *hcnBC*, restriction analysis was performed with the same four enzymes, and profiles were classified according to banding pattern (see Fig. 1).

ERIC-PCR, enterobacterial repetitive intergenic consensus-PCR.

[†]Genotype and cluster definitions of ERIC-PCR fingerprints are those of Fig. 3.

[‡]Estimated abundance (mean ± standard deviation; $n = 4$ plants) of genotypes based on their frequencies and total Phl$^+$ populations values determined in Ramette *et al*. (2003a).

[§]Production of pyoluteorin is indicated by a black diamond (◆) for each isolate.

NA, not available.

et al., 1989) composed of clay (10% weight in weight, w/w) and a mixture of quartz silt and sand (90% w/w), five aseptically grown 4-week-old tobacco plants, 10^5 fungal endoconidia cm^{-3} soil, 10^7 bacterial CFU cm^{-3} soil (when pseudomonads were used) and 3 mL of Knop nutrient solution to reach 20% w/w water content. After 3 weeks of plant growth in a growth chamber (70% air humidity; 16 h of daylight at 22 °C and 8 h of dark at 18 °C), disease severity was rated as the percentage of root necrosis (Stutz *et al*., 1986). Within each replicate, biocontrol performances (i.e. plant fresh weight or disease severity) of tested strains were expressed relative to those obtained with the reference strain *Pseudomonas* sp. CHA0 (i.e. as a ratio). The experiment was done twice, and data from both experiments were pooled after determining that interexperimental variation was not significant.

Statistical analyses

All experiments were done at least twice. The standard deviations presented in the study were obtained by pooling data from several experiments, after ensuring by ANOVA that variances were homogenous and inter-experimental variation not significant. When disease severity data were used raw (i.e. without dividing by data obtained with strain CHA0), an arcsine conversion was implemented to insure normality prior to performing statistical analyses. One-way and two-way ANOVA, Pearson correlation coefficient analysis followed by Bonferroni's significance test, and Fisher's least significant difference (LSD) tests were performed at $P < 0.05$, using SYSTAT (version 9, SPSS Inc., Chicago, IL).

Analyses of molecular variance (AMOVA) were performed with Arlequin (version 2.000; Schneider *et al*., 2000) to test

whether *Pseudomonas* populations originating from different soils were significantly different based on (i) *phlD* (using data in Ramette *et al*., 2003a) and *hcnBC* allelic frequency and (ii) diversity data obtained for these genes in Ramette *et al*. (2003a) and in this study. The pairwise genetic distance between isolates was measured as Euclidean distance based on the vectors of presence and absence of restriction bands. The significance of the variance components and associated *F* statistics for different population structures were evaluated using 10 000 non-parametric permutations (Excoffier *et al*., 1992). Pairwise F_{ST} statistics were used to assess the short-term genetic distances between *Pseudomonas* populations (Reynolds *et al*., 1983; Slatkin, 1995), and the significance of the rejection of nondifferentiation between populations was assessed using 3000 random permutations.

Results and discussion

Polymorphism of *phlD* and *hcnBC* biocontrol genes

Although more restriction enzymes were used here than in our previous study with *Pseudomonas* isolates from Morens (Ramette *et al*., 2003a), higher polymorphism for *phlD* was not evidenced (Table 2). This confirms our previous observations that restriction analysis of *phlD* using three restriction enzymes was effective enough to evidence most of the allelic polymorphism at that locus (Wang *et al*., 2001; Ramette *et al*., 2003a). The most discriminatory enzyme for *phlD* was *Hae*III (data not shown), which was already used in Ramette *et al*. (2003a).

Hence, additional biocontrol genes (*hcnBC*) were chosen to investigate allelic polymorphism. Like *phlD*, these genes are directly involved in the biocontrol ability of *Pseudomonas* sp. strain CHA0 against black root rot of tobacco (Keel *et al*., 1989; Voisard *et al*., 1989). *hcnBC* restriction analysis revealed up to 10 alleles, whereas only eight *phlD* alleles were found (Table 2). The most discriminatory enzyme for *hcnBC* was *Cfo*I (Fig. 1), which yielded seven different restriction profiles.

A good correspondence was found between *phlD* and *hcnBC* alleles (Table 2), and most isolates that clustered together based on *phlD* also clustered together (or close-by) when *hcnBC* was analyzed (Fig. 2). Noticeably, the nodal supports for both UPGMA-based dendrograms were generally weak for nodes located below a 50–60% similarity. This could explain why certain pseudomonads, such as Q2-87 and C10-181, were found at different locations in the two dendrograms. The positioning of *phlD* clusters HBNC, HBDC and FBDC lacked strong support, this time because the allelic similarity between them was high. Within those clusters, the position of C10-204 was however better resolved using *hcnBC* data, as indicated by a higher bootstrap value (79% vs. 57%; Fig. 2).

Pseudomonas isolates from both suppressive and conducive soils in Morens were found in three clusters containing two isolates or more, regardless of whether *phlD* or *hcnBC* was considered (Fig. 2). The *hcnBC* cluster AKOb consisted of four isolates from MC10 only. In the *phlD* tree, the latter isolates were clearly different from isolates from Morens suppressive soils. This confirms and extends (in the case of *hcnBC*) our previous observation made with *phlD* (Ramette *et al*., 2003a) that most biocontrol alleles can be evidenced in isolates from both Morens suppressive and conducive soils.

Genetic structure of Phl$^+$ HCN$^+$ *Pseudomonas* populations isolated from Morens soils

To examine the null hypothesis that no population differentiation occurs between Phl$^+$ HCN$^+$ *Pseudomonas* populations from suppressive and conducive soils, an AMOVA approach was used to partition molecular variance at different hierarchical levels based on *phlD* and *hcnBC* restriction data (Table 3). The AMOVA of the two concatenated loci did not significantly partition the total variance components between suppressive and conducive soils, and hence failed to reject the null hypothesis ($P > 0.05$). Only 25.4% of molecular variance was found between soils within suppressive or conducive categories, compared with 79% between individual soils, regardless of their suppressive status. Similar AMOVA results were obtained with *hcnBC* or *phlD* data separately, although *phlD* data generally gave *F* statistics of lower statistical significance compared with *hcnBC* (Table 3). This may be due to the lower allelic polymorphism found in *phlD* (Fig. 2; Table 2).

Therefore, AMOVA results indicated that most of the genetic differentiation occurs at the level of individual soils, and that the distinction between suppressive and conducive soils may not be relevant to explain the observed diversity. This suggests that long-term suppressiveness may not be attributable to qualitative and/or quantitative differences in the genetic diversity of Phl$^+$ HCN$^+$ pseudomonads between Morens suppressive and conducive soils.

To further determine which soils harbored the more differentiated Phl$^+$ HCN$^+$ *Pseudomonas* population, pairwise F_{ST} values were calculated to estimate the short-term genetic distance between populations of isolates from the four soils (Reynolds *et al*., 1983; Slatkin, 1995). Results indicated that the population from MC10 was significantly more differentiated than those from the three other soils ($P < 0.01$; 3000 permutations), and no significant differentiation was evidenced between the latter three soils (data not shown).

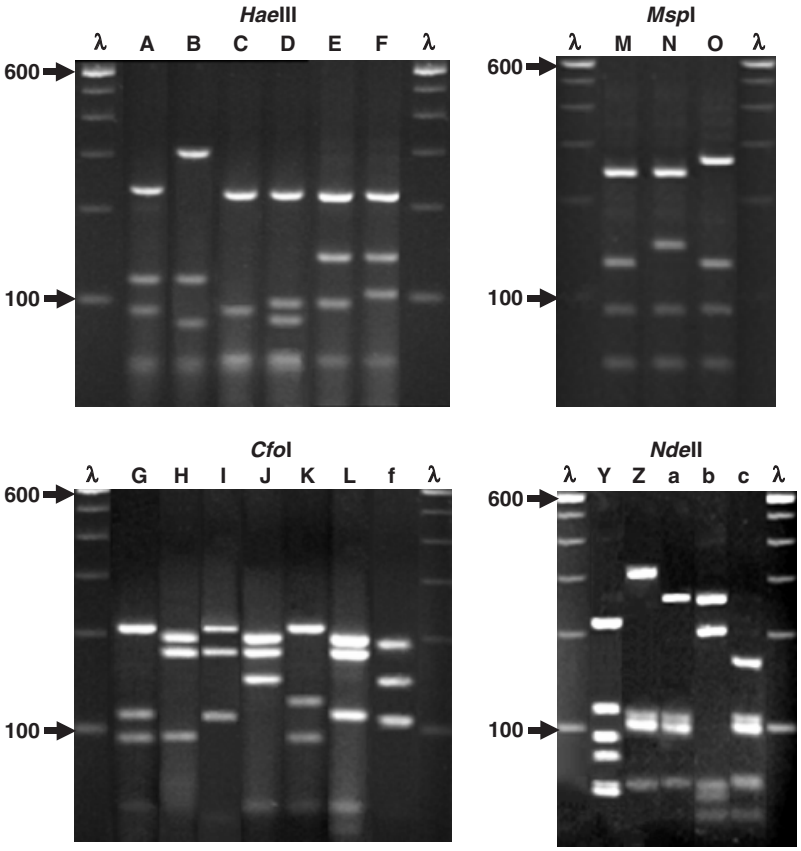

Fig. 1. Banding profiles of *hcnBC* obtained by digesting PCR amplicons from Morens pseudomonads with one of four endonucleases (*Hae*III, *Cfo*I, *Msp*I, *Nde*II). The profile denominations are the same as in Table 2.

Genetic diversity of Phl$^+$ HCN$^+$ pseudomonads at the strain level

The strain diversity of Morens isolates was determined using ERIC-PCR (Fig. 3). Most individual ERIC-PCR profiles were found in a single isolate only. When considering ERIC-PCR genotypes (i.e. at 95% similarity level), it appears that three genotypes (A, D and M; Fig. 3 and Table 2) were shared by two isolates or more. Interestingly, genotype D included isolates from MC6 only, while genotypes A (also that of reference strain CHA0) and M consisted of isolates from both suppressive and conducive soils. In the case of genotype M (Table 2), isolates S8-110 and C10-204 displayed the same ERIC-PCR genotype but different *phlD* and *hcnBC* alleles, which means that they were different strains.

Clusters were defined at an arbitrary 60% cut-off value (Fig. 3), yielding seven ERIC-PCR clusters, four of them gathering more than one isolate. Three of these four clusters included isolates from both conducive and suppressive soils. Perhaps these bacteria colonized the different field sites a long time ago and diverged into distinct, local genotypes, with no clear relationships with either the suppressive status of the soils investigated or their geographic location, as hypothesized previously (Ramette, 2002). Beyond the delineation of genotypes in our collection by ERIC-PCR, caution is however needed in interpreting evolutionary scenarios based on ERIC-PCR data. Indeed, it is known that there is no straightforward relationship between ERIC-PCR patterns and evolutionary rates in different bacterial lineages.

Although the same *phlD* and *hcnBC* alleles could be evidenced in both Morens suppressive and conducive soils, most of the isolates harboring them were genetically different based on ERIC-PCR. Therefore, it seems that Phl$^+$ *Pseudomonas* populations at Morens did not just consist of a few strains widely dispersed in the area under study, but of several highly differentiated strains. Previous observations based on phenotypic and *phlD* restriction analyses pointed to a cosmopolitan distribution of Phl$^+$ pseudomonads worldwide, especially for dicot-associated strains (Wang *et al.*, 2001), but the methods were not as resolutive as ERIC-PCR. Here, the use of population genetic analyses combined with a finer method to resolve microdiversity (i.e. ERIC-PCR) were necessary to evidence the existence of the genetic differentiation of *Pseudomonas* strains within each field site (based on AMOVA of restriction data) and significant levels of genomic heterogeneity between strains within and between sites, respectively. Similarly, fluorescent pseudomonads from pristine soils displayed an endemic distribution when using fingerprinting methods with the same level of

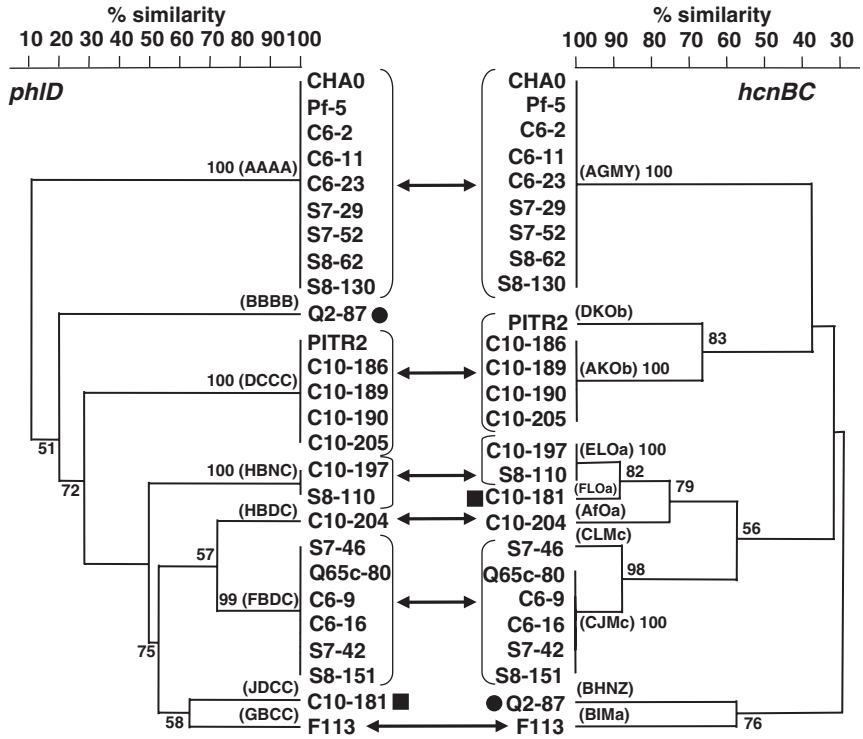

Fig. 2. Dendrograms inferred from *phlD* and *hcnBC* restriction data for Morens pseudomonads. The UPGMA clustering method was applied to a similarity matrix (Dice coefficient) that was based on presence/absence of restriction bands. Nodal support was assessed by 1000 bootstrap replicates, and only supports ≥50% are indicated. Allele designations (see Table 2) are indicated in brackets on each branch. Both the reference strain Q2-87 and the Morens isolate C10-181 showed different positions in *phlD* and *hcnBC*-based phylogenies, and they are signaled by ● and ■, respectively.

resolution as in the present study (i.e. BOX-PCR; Cho & Tiedje, 2000). The use of rep-PCR suggested substantial endemism for Phl^+ root-associated fluorescent pseudomonads in wheat monoculture (McSpadden Gardener *et al.*, 2000; Weller *et al.*, 2002; de Souza *et al.*, 2003), but $phlD^+$ populations in those soils displayed a lower *phlD* diversity than in Morens soils. Thus, the current work extends this concept of endemism to the Phl^+ HCN^+ fluorescent pseudomonads colonizing roots in farm soils subjected to crop rotation.

Biocontrol ability of Phl^+ HCN^+ pseudomonads *in vitro*

All Phl^+ HCN^+ *Pseudomonas* isolates significantly inhibited *T. basicola in vitro*, but to different extents ($P < 0.001$; Table 4). The data varied according to *phlD* and *hcnBC* alleles (Table 2). If one considers *hcnBC* alleles, isolates harboring the same allelic type as the reference strain CHA0 (i.e. AGMY) were more inhibitory than CHA0 itself (Table 4), whereas isolates harboring *hcnBC* alleles ELOa, AfOa or CLMc were less inhibitory than CHA0. For the other *hcnBC* alleles, different degrees of inhibition were observed. Noticeably, isolates with allele CJMc were more inhibitory than CHA0 when originating from suppressive soils (i.e. S7-42, S8-151), whereas those from conducive soils (i.e. C6-9, C6-16) were less inhibitory than CHA0 (Table 4). When isolates were grouped according to their soil of origin, no significant difference between soils could be detected (ANOVA; $P > 0.05$). Overall, there was therefore no relationship between either the origin of Morens isolates or their allelic types (*phlD* or *hcnBC*) and the *in vitro* suppression of the pathogenic fungus.

Biocontrol ability of Phl^+ HCN^+ pseudomonads *in planta*

At Morens, disease suppressiveness is linked to the prevalence of vermiculitic clays in suppressive soils (Keel *et al.*, 1989; Stutz *et al.*, 1989; Voisard *et al.*, 1989). When the biocontrol of the tobacco root pathogen *T. basicola* by Phl^+ HCN^+ pseudomonads from Morens was assessed, the plant weight data was significantly higher in vermiculite than in illite for all isolates but two, for which there was only a trend (i.e. C10-197 and C10-204) (Table 4). However, the difference between the two clay minerals was less pronounced when disease severity data were considered. Indeed, only five isolates (i.e. C6-9, C6-16, C10-190, C10-197 and S7-29) showed a significant difference between vermiculite and illite, and one of them (i.e. C10-197)

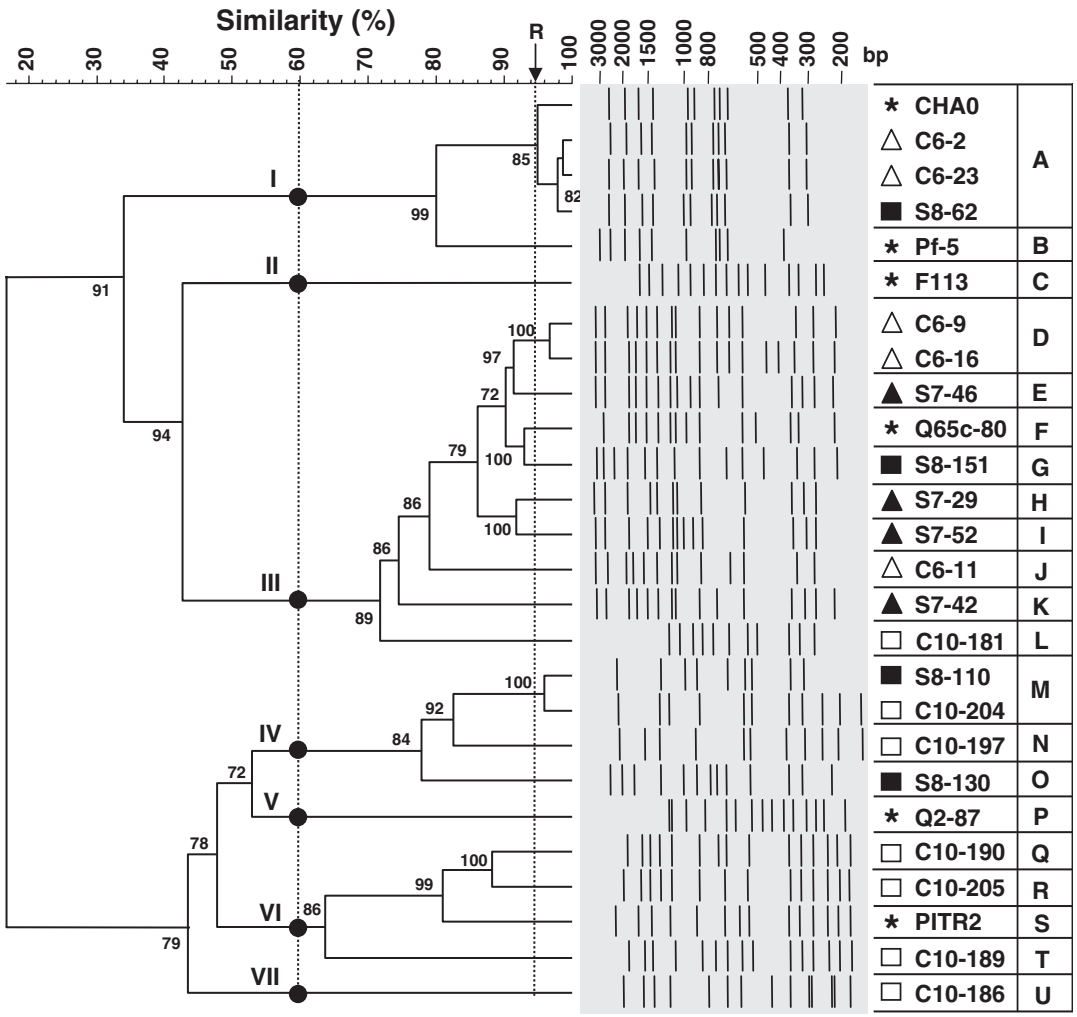

Fig. 3. Dendrogram and computer-generated profiles (GelCompar II) of enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprints of Morens pseudomonads. The UPGMA clustering method was applied to a similarity matrix calculated with the Dice coefficient. Consistency of each cluster was determined by the cophenetic correlation coefficient. The origin of the isolates is indicated as (*) for reference strains, (△, □) for conducive soils MC-6 and MC-10, respectively, and (▲, ■) for suppressive soils MS-7 and MS-8, respectively. Genotype definition is based on a cutoff value of 95%, representing the level of experimental reproducibility (R). Clusters are indicated by Roman figures, and correspond to an arbitrary cutoff value of 60% similarity.

had a higher disease severity in illite than in vermiculite (Table 4). This difference between the data of plant weight and disease severity was generally observed each time the experiment was run. This may be explained by the fact that significant weight differences due to pathogenic infection occur more rapidly than the apparition of chlamydospores and of fungal necrosis on the roots (our unpublished results).

There was however a strong correlation between plant weight and disease severity, regardless of isolate origin or the type of clay mineral (Table 6). The highest absolute values of correlation coefficients were always obtained in illite ($|r| \geq 0.67$; Table 6). However, the incorporation of disease severity as a covariate of plant weight in our statistical analyses did not change the significance of the aforementioned statistical results (data not shown).

Importance of isolate origin and clay mineralogy in biocontrol experiments in planta

A two-way ANOVA was performed to determine interactions between (i) the nature of the clay minerals used in the gnotobiotic systems and (ii) the soil origin of the isolates, i.e. suppressive vs. conducive soil (Analysis A) upon biocontrol performances (Table 5). The two factors did not show any significant interactions ($P > 0.05$), neither for plant weight nor disease severity. For the latter variable, there was no significant effect of classifying isolates based on the

Table 3. Analysis of population structure of Morens pseudomonads based on AMOVA of restriction data for *hcnBC* and *phlD*

	hcnBC			*phlD*			*hcnBC* + *phlD*		
Source of variation	Total (%)	*F*	*P*†	Total (%)	*F*	*P*	Total (%)	*F*	*P*
Between suppressive and conducive soils	− 3.9	− 0.039	NS	− 2.5	− 0.024	NS	− 4.8	− 0.048	NS
Between suppressive soils and between conducive soils	30.9	0.298	***	20.0	0.200	*	25.4	0.242	**
Between the four soils	73.0	0.270	***	82.5	0.176	*	79.4	0.206	**

F, Degree of differentiation at different hierarchical levels.
†Probability (*P*) of having significant variance components and associated *F*-statistics, as estimated by permutational analyses of the data matrices. *P* was not significant (NS), * < 0.05, ** < 0.01, *** < 0.001.

Table 4. Biocontrol performance of fluorescent pseudomonads from Morens. Data are expressed as a ratio, by dividing raw data by that obtained with the reference strain *Pseudomonas* sp. CHA0 (mean ± standard deviation)†

		Effect on plant weight‡		Effect on root disease severity§	
Isolates	Antagonistic effect *in vitro*¶	Vermiculitic soil	Illitic soil	Vermiculitic soil	Illitic soil
Conducive soils					
C6-2 ◆ ‖	1.1 ± 0.1 cd ↑	1.24 ± 0.07 a–c ↑	0.77 ± 0.10 j–m* ↓	1.42 ± 0.22 i–l	1.31 ± 0.43 kl
C6-9	0.6 ± 0.0 g ↓	1.26 ± 0.12 ab ↑	0.54 ± 0.09 no* ↓	1.46 ± 0.17 g–l	2.46 ± 0.39 b* ↑
C6-11 ◆	1.2 ± 0.1 a–c ↑	1.21 ± 0.21 b–d ↑	0.78 ± 0.19 i–m* ↓	1.62 ± 0.23 f–k ↑	1.72 ± 0.40 c–i ↑
C6-16	0.5 ± 0.1 g ↓	1.15 ± 0.25 b–f	0.64 ± 0.28 m–o* ↓	1.46 ± 0.41 g–l	1.98 ± 0.57 c–e* ↑
C10-181	1.0 ± 0.1 de	1.42 ± 0.18 a ↑	0.86 ± 0.13 h–l* ↓	1.70 ± 0.29 d–i ↑	1.72 ± 0.24 c–i ↑
C10-186	1.1 ± 0.0 cd	0.96 ± 0.08 f–i	0.77 ± 0.24 j–m* ↓	1.77 ± 0.39 c–g ↑	1.99 ± 0.32 c–d ↑
C10-190	1.3 ± 0.2 ab ↑	0.96 ± 0.07 f–i	0.53 ± 0.35 no* ↓	1.68 ± 0.34 d–j ↑	2.74 ± 0.55 ab* ↑
C10-197	1.0 ± 0.0 de	0.80 ± 0.12 i–m ↓	0.67 ± 0.25 m–o ↓	2.04 ± 0.30 c ↑	1.43 ± 0.45 h–l*
C10-204	0.8 ± 0.1 ef ↓	1.06 ± 0.15 c–g	0.93 ± 0.12 g–k	1.67 ± 0.27 d–j ↑	1.65 ± 0.27 e–j ↑
Suppressive soils					
S7-29 ◆	1.3 ± 0.0 ab ↑	1.06 ± 0.26 c–g	0.10 ± 0.17 p* ↓	1.72 ± 0.33 c–i ↑	2.80 ± 0.64 a* ↑
S7-42	1.3 ± 0.0 a ↑	1.18 ± 0.13 b–e	0.64 ± 0.20 m–o* ↓	1.26 ± 0.22 l	1.36 ± 0.35 j–l
S7-46	0.7 ± 0.1 fg ↓	1.03 ± 0.16 d–h	0.80 ± 0.13 i–m*	1.75 ± 0.28 c–h ↑	1.36 ± 0.14 j–l*
S7-52 ◆	1.1 ± 0.2 b–d ↑	1.08 ± 0.19 b–g	0.75 ± 0.08 k–m* ↓	1.86 ± 0.20 c–f ↑	1.83 ± 0.21 c–f ↑
S8-62 ◆	1.2 ± 0.1 a–c ↑	1.01 ± 0.07 e–h	0.69 ± 0.16 l–n* ↓	1.69 ± 0.29 d–j ↑	1.74 ± 0.37 c–i ↑
S8-110	0.8 ± 0.1 ef ↓	1.04 ± 0.13 d–h	0.76 ± 0.19 k–m* ↓	1.70 ± 0.19 d–i ↑	1.53 ± 0.23 f–l ↑
S8-130 ◆	1.2 ± 0.2 b–d ↑	1.10 ± 0.24 b–g	0.50 ± 0.17 o* ↓	2.04 ± 0.08 c ↑	2.55 ± 0.45 ab* ↑
S8-151	1.2 ± 0.0 b–d ↑	0.95 ± 0.16 g–k	0.81 ± 0.24 i–m ↓	1.74 ± 0.33 c–i ↑	1.75 ± 0.42 c–h ↑

†Raw data were divided by the values obtained with strain CHA0 within each replication, before computing means and standard deviations. For each isolate origin (i.e. conducive or suppressive soils), the letters next to the data indicate the statistical relationship between treatments (isolates) derived from ANOVA followed with Fisher's least significant difference tests ($P < 0.05$). In addition, significant differences between the two types of soil (i.e. vermiculitic and illitic soils) for a given isolate are shown using*. Significant differences between an isolate and the reference strain CHA0 are shown using ↑ and ↓ for significant increase or decrease, respectively.
‡Total fresh weight (mg) was recorded for each of five plants in each treatment. Plant fresh weight data for strain CHA0 were 110 ± 14 mg in vermiculitic soil and 104 ± 19 mg in illitic soil.
§Disease level was determined for each of five plants in each treatment, using an eight-class disease scale (Stutz *et al*., 1986). Disease scores for strain CHA0 were 30 ± 9% in vermiculitic soil and 31 ± 11% in illitic soil.
¶*T. basicola* growth inhibition was measured as the distance between the bacterial colony and fungal mycelium on plate ($n = 3$). Data for strain CHA0 was 7.0 ± 0.4 mm.
‖Production of pyoluteorin is indicated by a black diamond (◆) for each isolate.

suppressive or conducive status of the soil of origin, but overall significantly lower ($P < 0.001$) disease severity levels were obtained with isolates in vermiculite than in illite. Unexpectedly, plant weight was statistically higher for isolates from conducive soils (99.8 ± SD 33.8 mg) than for those from suppressive soils (90.7 ± 35.6 mg). It was also higher in vermiculite (119.5 ± 23.1 mg) than in illite (70.5 ± 26.4 mg). This can be partly explained by rather low values in illite for isolates from suppressive soils (e.g. S7-29, S8-130; Table 4).

The two-way ANOVA was also performed with a comparison of the four soils (Analysis B). This time, a significant

Table 5. Effects of the origin of Morens isolates and of clay minerals upon biocontrol activity ($n = 218$)

	Plant weight (mg)			Disease severity (%)		
Factor*	*F* ratio	df†	*P*‡	*F* ratio	df	*P*
Analysis A						
Isolate origin (suppressive vs. conducive soil)	6.12	1	**	1.74	1	NS
Clay mineral in the assay (vermiculite vs. illite)	217.27	1	***	18.77	1	***
Isolate origin × clay mineral type	0.90	1	NS	0.20	1	NS
Analysis B						
Isolate origin (soils MC6, MC10, MS7, MS8)	2.77	3	*	0.99	3	NS
Clay mineral in the assay (vermiculite vs. illite)	238.79	1	***	19.41	1	***
Isolate origin × clay mineral type	5.87	3	***	1.47	3	NS

*Two-way ANOVA were performed by considering either two (suppressive and conducive soils; Analysis A) or four soil categories (the four soils; Analysis B) for the origin of the Morens isolates. Statistical analyses were done on raw data for each strain.

†Degrees of freedom (df).

‡*P* values are represented by (NS) when not significant, *$P < 0.05$, **when $P < 0.01$, ***when $P < 0.001$.

Table 6. Significant correlations between plant fresh weight (mg) and root disease severity (%) in the tobacco/*T. basicola* biocontrol assay*

Description of the analysis	*n*†	*r*‡
All isolates		
Data from both vermiculitic and illitic soils	218	− 0.64
Data from vermiculitic soil	111	− 0.39
Data from illitic soil	107	− 0.78
Isolates from suppressive soils		
Data from both vermiculitic and illitic soils	104	− 0.62
Data from vermiculitic soil	52	− 0.28
Data from illitic soil	52	− 0.86
Isolates from conducive soils		
Data from both vermiculitic and illitic soils	114	− 0.58
Data from vermiculitic soil	59	− 0.45
Data from illitic soil	55	− 0.67

*There was no correlation between *in vitro* inhibition of *T. basicola* and either disease severity in vermiculitic soil, disease severity in illitic soil, plant fresh weight in vermiculitic soil, or plant fresh weight in illitic soil ($n = 20$).

†Number of comparisons used.

‡Pearson's correlation coefficient (r). Statistical analyses were done on raw data for each strain. All correlations were significant at $P < 0.001$ (Bonferroni's significance tests).

interaction between the two factors was observed for plant weight, but not disease severity (Table 5). For the latter variable, two-way ANOVA results were very similar with those in Analysis A, indicating that, overall, more healthy roots were found in vermiculite than in illite. For plant weight, both the specific soil origin of the isolates and the type of clay mineral had an influence. In vermiculite, inoculation with isolates from MC6 (133.2 ± 11.5 mg) led to significantly higher plant weight than with MC10 (113.6 ± 27.6 mg) or MS8 (112.6 ± 17.6 mg) ($P < 0.05$; Fisher LSD test). Isolates from MS7 (121.4 ± 19.8 mg) had an intermediary position, being not significantly different from other soil isolates. The situation in illite was different than in vermiculite since a significantly lower plant weight was obtained with isolates from MS7 (57.4 ± 32.3 mg) than with isolates from the three other soils ($P < 0.05$). Among the isolates from the latter three soils, there was no statistical difference (MC6, 71.1 ± 19.0 mg; MC10, 80.2 ± 25.9 mg; MS8, 72.4 ± 21.9 mg; $P > 0.05$). This indicates that biocontrol performances may differ when isolates are grouped according to their particular soil origin, but not when grouped according to the suppressiveness status of the soil.

Summarizing, those analyses support the concept that Phl^+ pseudomonads isolated from conducive soils may be as good or even better biocontrol agents than Phl^+ isolates from suppressive soil, when the former are placed under biocontrol-favorable conditions. Conversely, under environmental conditions which limit biocontrol activity, putative biocontrol strains from suppressive soils may perform as badly as or even worse than Phl^+ isolates from conducive soil.

Pyoluteorin production by Phl^+ HCN^+ pseudomonads

The Phl precursor monophloroglucinol was produced by all Phl^+ isolates, as expected, whereas Plt production was an exclusive property of all Phl^+ HCN^+ isolates harboring *phlD* allele AAAA (and *hcnBC* allele AGMY). These pseudomonads correspond to the 16S rRNA gene restriction group designated ARDRA-1, to which the Plt^+ reference strains CHA0 and Pf-5 belong (Keel *et al.*, 1996; Ramette *et al.*, 2003b). They displayed several different ERIC-PCR genotypes (Table 2), which is interesting because previous

attempts to distinguish them genetically from one another were met with little success (Sharifi-Tehrani *et al.*, 1998).

In both soils, Plt^+ Phl^+ HCN^+ pseudomonads (based on data from six strains, i.e. C6-2, C6-11, S7-29, S7-52, S8-62 and S8-130) led to significantly lower plant weight overall ($P = 0.009$) than Plt^- Phl^+ HCN^+ isolates, while no significant difference was observed between the two groups of Swiss isolates for disease severity ($n = 6$; ANOVA). This confirms previous findings from the tomato/Fusarium crown and root rot pathosystem (Sharifi-Tehrani *et al.*, 1998). Indeed, although Plt itself displays antifungal properties (Howell & Stipanovic, 1979; Haas & Keel, 2003), it also acts as a repressor of Phl biosynthesis (Schnider-Keel *et al.*, 2000).

Conclusions

No clear demarcation was found between Phl^+ HCN^+ *Pseudomonas* isolates from suppressive and conducive soils in terms of the presence of particular biocontrol alleles and biocontrol performances. This is not totally surprising, because Phl production has been advocated as a good marker for the selection of biocontrol strains (Sharifi-Tehrani *et al.*, 1998; Moënne-Loccoz & Défago, 2004). The fact that Phl^+ HCN^+ pseudomonads with antagonistic potential (Table 4) were found at similar, high-population densities in both Morens suppressive and conducive soils (Ramette *et al.*, 2003a) means that a different explanation is needed for the disease-suppressive status of Morens soils compared with other types of suppressive soils where Phl^+ pseudomonads are implicated. Indeed, in the case of induced suppressiveness to take-all, Phl^+ pseudomonads exceed a threshold population density in contrast to conducive situations (Raaijmakers & Weller, 1998; de Souza *et al.*, 2003).

Less plant protection is generally observed in Morens conducive soils than in their suppressive counterparts, and here isolates from conducive soils protected plants to a similar extent as isolates from suppressive soils did, when present in iron-rich vermiculite soil that mimicks Morens suppressive soil (i.e. favorable to biocontrol). This could mean that the expression of biocontrol genes is hampered in conducive soils, which is compatible with previous findings on the effects of abiotic factors on the production of biocontrol compounds in pseudomonads (Duffy & Défago, 1999). Further work is thus needed to test this hypothesis in rhizosphere situations.

This work was based on the assumption that the long-standing suppressiveness of Morens soils to black root rot of tobacco was due mainly to antagonistic fluorescent pseudomonads (Stutz *et al.*, 1986). An alternate hypothesis that is compatible with the current results is that Morens soil suppressiveness could also involve other plant-protecting microbial populations (so far unidentified), whose role in disease suppressiveness may be as important, or perhaps even more important, than the antagonistic activity of biocontrol pseudomonads.

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